

Free radical scavenging capacity and antioxidant enzyme activity in deerberry (*Vaccinium stamineum* L.)

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Abstract

Fruit from three genotypes ('B-76', 'B-59' and 'SHF-3A') of deerberry [*Vaccinium stamineum* L.] were evaluated for fruit quality, total anthocyanin and phenolic contents, antioxidants, antioxidant capacity, and antioxidant enzyme activity. The fruit soluble solids, titratable acids, total anthocyanins, and total phenolic contents varied with genotypes. Cyanidin 3-galactoside and cyanidin 3-arabinoside were the two predominant anthocyanins. Resveratrol was also found in deerberries. Among the three genotypes, 'B-76' had higher amount of anthocyanins, phenolic compounds and resveratrol than 'B-59' and 'SHF-3A'. Deerberries contained potent free radical scavenging activities for 2,2-Di (4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH[•]), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS^{•+}), peroxy radical (ROO[•]), superoxide radicals (O₂^{•-}), hydrogen peroxide (H₂O₂), hydroxyl radicals ([•]OH), and singlet oxygen (¹O₂) radicals and also had high activities of antioxidant enzymes including glutathione-peroxidase (GSH-POD), glutathione reductase (GR), superoxide dismutase (SOD), ascorbate peroxidase (AsA-POD), guaiacol peroxidase (G-POD), monodehydroascorbate radical reductase (MDAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR)] and non-enzyme antioxidants [ascorbic acid (ASA) and reduced glutathione (GSH)]. Antioxidant capacities were highly correlated to antioxidant enzymes activities. Among the three genotypes, 'B-76' had the highest level of antioxidants and antioxidant enzyme activity. © 2006 Swiss Society of Food Science and Technology. Published by Elsevier Ltd. All rights reserved.

Keywords: *Vaccinium stamineum*; Sugar; Acid; Anthocyanin; Phenolics; Radical scavenging; Antioxidant enzyme

1. Introduction

Antioxidants are compounds that can delay or inhibit the oxidation of lipid or other molecules by inhibiting the initiation or propagation of oxidizing chain reactions (Velioglu, Mazza, Gao, & Oomah, 1998). Berry fruits are good sources of natural antioxidants. In addition to the usual nutrients such as vitamins and minerals, berries are also rich in anthocyanins, flavonoids and phenolic acids (Heinonen, Meyer, & Frankel, 1998; Velioglu et al., 1998). The antioxidant activity of phenolic compounds are mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides and have health functional properties that may protect humans

from various diseases (Heinonen et al., 1998; Rice-Evans & Miller, 1998). Consumption of berry fruits has been associated with lower incidence and mortality rates of cancer in several human cohort and case-control studies (Heinonen et al., 1998; Rice-Evans & Miller, 1996). A high intake of berry fruits has also been reported to prevent urinary tract infections, enhance the immune function, reduced blood pressure and cardiovascular diseases (Ames, Shigena, & Hagen, 1993; Ascherio et al., 1992).

Deerberry (*Vaccinium stamineum* L.) is a low; Slender shrub. The fruit of deerberry resemble cranberries (*V. macrocarpum* Ait.) and have been utilized for flavored jelly; Preserves and pie (Ballinger, Maness, & Ballington, 1981). Deerberry fruit contain high amount of anthocyanins and phenolic compounds (Ballington, Kirkman, Ballinger, & Maness, 1988; Rimando, Kalt, Magee, Dewey, & Ballington, 2004). These naturally occurring compounds have been shown to have health benefits (Heinonen et al.,

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1998; Rice-Evans & Miller, 1996). However, being a minor crop, Deerberries received little attention for research and little information is available on the health benefits. The purpose of this study is to evaluate the antioxidant capacity and the antioxidant enzymes activity that might be involved in oxygen detoxification in three genotypes of deerberry (B-59; B-76 and SHF3A-3:127).

2. Materials and methods

2.1. Chemicals

Ascorbate, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS^{•+}), histidine, hydrogen peroxide (30% w/w), hydroxylamine hydrochloride, *N,N*-dimethyl-*p*-nitrosoaniline, xanthine, xanthine oxidase, dithiothreitol (DTT), glutathione (oxidized form), glutathione (GSH, reduced form), glutathione reductase, guaiacol, β -nicotinamide adenine dinucleotide (β -NADH, reduced form), β -nicotinamide adenine dinucleotide phosphate (β -NADPH, reduced form), nitro blue tetrazolium (NBT), resveratrol (3,5,4'-trihydroxystilbene), and FeSO₄ were purchased from Sigma Chemical Co. (St. Louis, MO), EDTA (ethylenediaminetetracetic acid, disodium salt, dihydrate-Na₂ EDTA · 2H₂O),

6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and 2,2-Di (4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH), and trichloroacetic acid were purchased from Aldrich (Milwaukee, WI). 2', 2' Azobis (2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA Inc. (Richmond, VA).

2.2. Fruit sample preparation

Deerberry fruit (*Vaccinium stamineum* L.) used in this study were grown at Jackson Spring, NC, USA, and were hand-harvested at a commercially mature stage. The fruits were sorted to eliminate damaged, shriveled, and unripe fruit, and selected for uniform size and color. Undamaged berries were randomized and frozen in liquid nitrogen, and then stored at -80°C until they were used for analysis. For the assays of anthocyanins, total phenolics, flavonoids, oxygen radical absorbance capacity (ORAC), 2,2-Di (4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH) and cation 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS^{•+}) scavenging activity, triplicate deerberries (2 g) from each genotype were extracted with 20 ml 80% acetone (containing 0.2% formic acid) using a Polytron (Brinkmann Instruments, Inc., Westbury, NY). The homogenized samples from the acetone extraction were then centrifuged at $14,000 \times g_n$ for 20 min at 4°C . The supernatants were transferred to vials, stored at -80°C , and later used for analysis.

To prepare the samples for superoxide radicals ($\text{O}_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^{\bullet}), and singlet oxygen ($^1\text{O}_2$) assay, three-100 g samples of deerberries from each genotype were pulverized and then

centrifuged at $14,000 \times g_n$ for 20 min at 4°C . The supernatants were transferred to vials stored at -80°C until used for analysis.

2.3. Analysis of soluble solids content (SSC) and titratable acids (TA)

Deerberries were pulverized with a cold mortar and pestle and pressed through four layers of cheese cloth to express the juice used for SSC and TA determination. The SSC of the fruit was determined on a digital refractometer Palette 100 PR-100 (ATAGO-Spectrum Technologies, Plainfield, ILL.) standardized with distilled water. TA was determined by diluting each 5-ml aliquot of lingonberry juice to 100 ml with distilled water and adjusting the pH to 8.2 using 0.1 N NaOH. Acidity was expressed as percent of citric acid equivalent.

2.4. Total anthocyanin and total phenolic content

Total anthocyanin content in deerberries extracts was determined using the pH differential method (Cheng & Breen, 1991). Results were expressed as milligrams of cyanidin-3-galactoside equivalent per kg of fresh weight. Total soluble phenolics in the fruit extracts were determined with Folin–Ciocalteu reagent by the method of Slinkard and Singleton (1977) using gallic acid as a standard. Results were expressed as mg gallic acid equivalent (GAE) per kg of fresh weight.

2.5. HPLC analysis of anthocyanins, phenolic compounds and resveratrol

Fruit samples of 4 g deerberry fruit were extracted twice with 20 ml of 80% acetone–0.2% formic acid using a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY) for one min. Extracts were combined and concentrated to 1 ml using a Buchler Evapomix (Fort Lee, NJ) in a water bath at 30°C . The concentrated samples were dissolved in 4 ml of acidified water (3% formic acid) and then passed through a C₁₈ Sep-Pak cartridge (Waters), which was previously activated with methanol followed by water and then 3% aqueous formic acid. Anthocyanins and other phenolics were then recovered with 2.0 ml of acidified methanol containing 3% formic acid. The methanol extract was passed through a 0.45- μm membrane filter (Millipore, MSI, Westboro, MA) and 25 μl was analysed by HPLC. The separation and identification of individual anthocyanin and phenolic compound were performed with a HPLC following the procedures described previous by Wang and Zheng (2005). Identification and quantification of anthocyanins and phenolics was carried out according to methods previously reported (Wang & Zheng, 2005). The *trans*-resveratrol in the purified extracts was injected to a HPLC system as described Lamuela-Raventos, Romero-Perez, Waterhouse, and de la Torre-Boronat (1995). Results of the analyses for resveratrol are

expressed as microgram of *trans*-resveratrol per kg of fresh weight.

2.6. Free radical measurements

2.6.1. 2,2-Di (4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH) assay

The method described by Hatano, Kagawa, Yasuhara, and Okuda (1988) was used for determining the antioxidant activity of deerberries extracts on scavenging 2,2-Di (4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH) radicals. The decrease in absorbance was measured at 515 nm against a blank without extract using a spectrophotometer. Using a calibration curve with different amounts of DPPH, the ED₅₀ was calculated. The ED₅₀ is the concentration of an antioxidant that is required to quench 50% of the initial DPPH radicals under the experimental conditions given.

2.6.2. Radical cation 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS^{•+}) scavenging activity

Radical cation scavenging capacity of deerberry extract was examined against ABTS^{•+} generated by chemical methods (Miller & Rice-Evans, 1997). The ABTS^{•+} was prepared by oxidizing a 5 mmol/l aqueous solution of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) with manganese dioxide at ambient temperature for 30 min. The assay reaction contained 1.0 ml of ABTS^{•+} with an absorbance of 0.7 at 734 nm, 100 µl of deerberry acetone extract and 1.0 ml of 5 mmol/l PBS buffer (pH 7.4). Absorbance at 734 nm was measured after 1 min of reaction time, and the Trolox equivalent was calculated using a standard curve prepared with Trolox.

2.6.3. Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was carried out using a high-throughput instrument platform consisting of a robotic eight-channel liquid handling system and a microplate fluorescence reader (Huang, Ou, Hampsch-Woodill, Flanagan, & Prior, 2002). Final ORAC values were calculated using the regression equation between Trolox concentration and the net area under the curve (AUC) and were expressed as mmol Trolox equivalents per kg fresh weight.

2.6.4. Superoxide radical (O₂^{•-}) assay

The assay for superoxide radical (O₂^{•-}) was determined using the methods of Richmond, Halliwell, Chauhan, and Darbre (1981). The O₂^{•-} was generated by xanthine xanthine-oxidase systems. The final results were expressed as percent inhibition of O₂^{•-} production in the presence of fruit extract. The antioxidant capacity of fruit extract against O₂^{•-} value was expressed as mmole of ascorbate equivalent per kg fresh weight.

2.6.5. Hydrogen peroxide (H₂O₂) assay

The assay for hydrogen peroxide in fruit extracts of strawberry was carried out following procedures previously described by Patterson, MacRae, and Ferguson (1984).

The antioxidant capacity of fruit extract against H₂O₂ value was expressed as mmole of ascorbate equivalent per kg fresh weight.

2.6.6. Hydroxyl radical (•OH) assay

The assay for hydroxyl radical (•OH) was determined using the methods of Richmond et al. (1981). The •OH in aqueous media is generated through the Fenton reaction. Relative scavenging efficiency (% inhibition of hydroxylation) of fruit extract was estimated from the difference in absorbance (OD) with and without addition of the fruit extract. The antioxidant capacity of fruit extract against •OH value was expressed as mmole of ascorbate equivalent per kg fresh weight.

2.6.7. Singlet oxygen (¹O₂) assay

The production of singlet oxygen (¹O₂) by sodium hypochloride and H₂O₂ was determined by using a spectrophotometric method according to Chakraborty and Tripathy (1992) with minor modifications in which *N,N*, dimethyl-*p*-nitrosoaniline was used as a selective scavenger of ¹O₂ and histidine as a selective acceptor of ¹O₂. The bleaching of *N,N*, dimethyl-*p*-nitrosoaniline was monitored spectrophotometrically at 440 nm. Relative scavenging efficiency (% inhibition production of ¹O₂) of fruit extract was estimated from the difference in absorbance of *N,N*, dimethyl-*p*-nitrosoaniline with and without the addition of fruit extract. The antioxidant capacity of fruit extract against ¹O₂ value was expressed as mmole of ascorbate equivalent per kg fresh weight.

2.7. Antioxidant enzyme measurements

2.7.1. Glutathione-peroxidase (GSH-POD, EC 1.11.1.9), and glutathione reductase (GR, EC 1.6.4.2)

Fruit tissues (10 g fresh weight) were homogenized in 10 ml 0.1 mol/l Tris-HCl buffer (pH 7.8) containing 2 mmol/l EDTA-Na, 2 mmol/l dithiothreitol (DTT). The homogenate was centrifuged at 20,000 × *g*_n for 30 min at 4 °C, and the supernatant was used for the GSH-POD and GR assays. GSH-POD activity was determined using the method of Tappel (1978) with a slight modification. The reaction mixture contained 0.1 mol/l Tris-HCl buffer (pH 8.0), 0.4 mmol/l EDTA, 1.0 mmol/l NaN₃, 1.0 mmol/l H₂O₂, 1.0 mmol/l glutathione (GSH), 0.15 mmol/l NADPH, 1 unit of glutathione reductase and 100 µl enzyme extract. The total reaction volume was 1.0 ml. H₂O₂ was added to start the reaction. GSH-POD activity was determined by the rate of NADPH oxidation at 340 nm via a spectrophotometer (Shimadzu UV-160A, Shimadzu Scientific Instruments, Columbia, MD). Enzyme activity was expressed as nmole of NADPH oxidized per mg of protein per min.

GR activity was assayed according to Smith, Vierheller, and Thorne (1988). The activity of GR was determined by monitoring glutathione-dependent oxidation of NADPH at 340 nm. GSSG was added to start the reaction and the

rate of oxidation was calculated using the extinction coefficient of NADPH ($6.22 \text{ mmol/l}^{-1} \text{ cm}^{-1}$). GR activity was expressed as nmole of NADPH oxidized per mg of protein per min.

2.7.2. Superoxide dismutase (SOD, EC 1.15.1.1)

Fruit tissues (10 g) were pulverized in a cold mortar and pestle with 10 ml K-phosphate buffer (0.1 M, pH 7.3) containing 1 mmol/l EDTA, 2 mmol/l DTT. The homogenate was strained through 4 layers of miracloth and centrifuged at $12,000 \times g_n$ for 10 min at 4°C . The supernatant was purified according to Wang, Jiao, and Faust (1991) before assaying the SOD enzyme activity.

Total SOD activity was assayed photochemically. Dicoumarol was included in the reaction mixture to inhibit reduction by pyridine nucleotide and to obtain a completely $\text{O}_2^{\bullet-}$ -dependent reduction of NBT. One unit of SOD was defined as the amount of enzyme which produced a 50% inhibition of NBT reduction under assay conditions. Since inhibition is not linearly correlated with SOD concentration, a V/v transformation was used to obtain linearity (V = basic reaction rate without deerberry fruit extract, v = reaction rate with extract). Linear correlation gave the equation: SOD units/ml = $(0.459 V/v - 0.032) \times \text{dilution factor}$.

2.7.3. Ascorbate peroxidase (AsA-POD, EC 1.11.1.11), and Guaiacol peroxidase (G-POD, EC 1.11.1.7)

Fruit tissues (10 g) were pulverized in a cold mortar and pestle with 10 ml K-phosphate buffer (0.1 mol/l, pH 7.3) containing 1 mmol/l EDTA, 2 mmol/l DTT. The homogenate was centrifuged at $12,000 \times g_n$ for 10 min at 4°C . The supernatant was used for the AsA-POD, and G-POD assays.

AsA-POD activity was assayed according to the method of Amako, Chen, and Asada (1994). H_2O_2 was added to start the reaction. Enzyme activity was expressed as nmole of ascorbate oxidized per mg of protein per min.

The G-POD assay mixture contained 0.1 mol/l phosphate buffer (pH 6.1), 4 mmol/l guaiacol as donor, 3 mmol/l H_2O_2 as substrate and 1.0 ml crude enzyme extract. The total reaction volume was 3.0 ml. The rate of change in absorbance at 420 nm was measured, and the level of enzyme activity was expressed as the difference in absorbance (OD) per mg protein, per min.

2.7.4. Dehydroascorbate reductase (DHAR, EC 1.8.5.1)

DHAR activity was assayed by measuring the rate of NADPH oxidation at 340 nm (Shigeoka, Nakano, & Kitaoka, 1980). The reaction mixture contained 50 mmol/l potassium phosphate (pH 6.1), 0.2 mmol/l NADPH, 2.5 mmol/l dehydroascorbate, 2.5 mmol/l glutathione, 0.6 unit glutathione reductase (GR; from spinach, EC 1.6.4.2) and 0.1 ml of diluted fruit juice (2 ml juice was diluted with 2 ml 50 mmol/l potassium phosphate, pH 6.1). The reaction was started by adding dehydroascorbate. Enzyme activity was expressed as nanomoles of NADPH oxidized per milligram of protein per min.

2.7.5. Monodehydroascorbate reductase (MDAR, EC 1.6.5.4)

MDAR activity was assayed by measuring the rate of NADH oxidation at 340 nm (Nakagawara & Sagisaka, 1984). The reaction mixture contained 50 mmol/l K-phosphate buffer (pH 7.3), 0.2 mmol/l NADH, 1.0 mmol/l ascorbate, 1.0 unit of ascorbate oxidase and 0.1 ml of 50 mmol/l K-phosphate buffer (pH 7.3) diluted fruit juice (2–1 dilution) in a total volume of 1.0 ml. The reaction was started by adding ascorbate oxidase (from *Cucurbita*, EC 1.10.3.3). Enzyme activity was expressed as nanomoles of NADH oxidized per milligram of protein per min.

2.8. Determination of ascorbate (AsA) and glutathione (GSH)

For measurement of AsA, fruit samples of 4 g were homogenized with a cold mortar and pestle using 8 ml ice-cooled 5% trichloroacetic acid (TCA). The homogenate was filtered through four layers of miracloth and centrifuged at $16,000 \times g_n$ for 10 min at 4°C . The supernatant was used for the AsA assays. AsA was determined using the methods of Arakawa, Tsutsumi, Sanceda, Kurata, and Inagaki (1981). A standard curve in the range 0–10 μmol AsA was used.

For measurement of GSH, triplicate deerberry fruit samples of 4 g were homogenized in 8.0 ml ice-cold, degassed 7.57 mmol/l sodium ascorbate solution with chilled mortar and pestle under N_2 at 0°C . The homogenate was filtered through four layers of miracloth and centrifuged at $30,000 \times g_n$ for 15 min at 0°C . The supernatant was deproteinized in glass test tubes by incubation in a water bath at 100°C for 3 min and then centrifuged at $15,000 \times g_n$ for 15 min at 0°C . The supernatants were used for the GSH assay. GSH was assayed using the method described by Castillo and Greppin (1988).

2.9. Protein determination

Protein was determined according to Bradford (1976), using bovine serum albumin (BSA) as a standard.

2.10. Statistical analysis

All experiments were conducted three times independently. Samples from different replicates were stored separately at -80°C until analysed. Results were given as mean \pm standard deviation of six independent determinations. All statistical analyses were performed with NCSS Statistical Analysis System (Statistical System for Windows, Kaysville, UT, USA) (NCSS, 2000). One-way analysis of variance (ANOVA) was used to compare the means. Differences were considered significant at $P \leq 0.05$. Correlation and regression analyses of free radical scavenging capacity (ROO^\bullet , $^\bullet\text{OH}$, H_2O_2 , $\text{O}_2^{\bullet-}$ and $^1\text{O}_2$) versus activities of antioxidant enzymes (SOD, G-POD, GSH-POD, AsA-POD, MDAR, DHAR, and GR) and the

non-enzyme components (AsA, DHAsA, GSH, GSSG) were also performed using NCSS (NCSS 2000, Kaysville Utah) (2000).

3. Results

SSC in three genotypes of deerberries ranged from 14.7% to 16.8% and TA ranged from 0.10% to 0.21% (Table 1). Genotype SHF3A-3:127 had the highest SSC and TA and 'B-76' had the highest total anthocyanin and total phenolics. Higher total anthocyanin and total phenolic contents were obtained when the fruit were extracted with acetone than when analyzed directly from fruit juices.

HPLC analysis of deerberries acetone extract showed that, in addition to anthocyanins, other phenolic compounds were present in significant amounts (Table 2). Compounds such as caffeic acid, *p*-coumaric acid, querce-

tin 3-galactoside, quercetin 3-rhamnoside, quercetin derivative and kaempferol were detected. Caffeic acid ranged from 40.6 to 61.2 mg/kg fresh wt (fw) and *p*-coumaric acid ranged from 27.7 to 33.1 mg/kg fw. Flavonols such as cyaniding 3-galactoside, cyaniding 3-glucoside, cyaniding 3-arabinoside and peonidin 3-glucoside in deerberries ranged from 32.2 to 987.5 mg/kg fw. Cyanidin 3-galactoside and cyaniding 3-arabinoside were the two predominant anthocyanins. Among the three genotypes, 'B-76' had higher amounts of anthocyanins and phenolic compounds than the other two genotypes except that 'SHF3A-3:127' had the highest amount of peonidin 3-glucoside (Table 2).

Resveratrol (3,5,4'-trihydroxystilbene) was also detected in deerberries. The value of resveratrol for deerberry 'B-59', 'B-76' and 'SHF3A-3:127' were 47.9, 68.5 and 36.2 µg/kg fw, respectively (Table 2).

Deerberries had high antioxidant capacity against radicals of DPPH, ABTS^{•+}, ROO[•], O₂^{•-}, H₂O₂, [•]OH,

Table 1
Soluble solids content (SSC), titrable acidity (TA), anthocyanins, total phenolics and antioxidant activity (ORAC, ABTS and DPPH) in three genotypes of deerberries^a

	B-59	B-76	SHF3A-3:127
SSC (%)	14.7±0.8	16.4±0.5	16.8±0.5
TA (%)	0.10±0.02	0.11±0.04	0.21±0.02
Anthocyanins ^b (mg/kg fw)			
Juice	508±1.5	954±12.9	720±11.8
Acetone extract	3714±34.4	6306±36.1	4696±115
Total phenolics ^c (mg/kg fw)			
Juice	1827±107	2939±127	2135±118
Acetone extract	8573±131	9654±190	8836±209
Antioxidant activity			
ORAC ^d (mmol TE/kg fw)			
Juice	50.3±1.05	68.5±2.86	61.9±1.23
Acetone extract	152.5±11.2	173.4±12.3	164.6±10.7
ABTS ^d (mmol TE/kg fw)	2.28±0.09	2.92±0.26	2.51±0.10
DPPH-radical scavenging activity			
ED ₅₀ ^e (mg fw)			
Juice	7.40±0.05	6.48±0.56	7.34±0.04
Acetone extract	0.43±0.17	4.47±0.38	5.24±0.15
Significance ^f	Extraction (juice vs. acetone)	Genotype	
SSC	n/a	*	
TA	n/a	*	
Anthocyanin	*	*	
Total phenolics	*	*	
ORAC	*	*	
ABTS	n/a	*	
DPPH-radical scavenging activity			
ED ₅₀	*	*	

^aData expressed as mean±standard deviation, *n* = 6.

^bData expressed as mg of cyanidin 3-glucoside equivalents per kg of fresh weight.

^cData expressed as mg of gallic acid equivalents per kg of fresh weight.

^dData expressed as mmoles of Trolox equivalents per kg of fresh weight.

^eThe ED₅₀ is that concentration of an antioxidant (mg fresh weight of deerberries) which is required to quench 50% of the initially DPPD radicals under the experimental conditions given.

^f*, significant at *P*≤0.05.

and $^1\text{O}_2$ (Tables 1 and 3). The scavenging activity for ROO^\bullet radical is expressed as ORAC activity. Significant differences were found among three deerberry genotypes in scavenging DPPH, $\text{ABTS}^{\bullet+}$, ROO^\bullet , $\text{O}_2^{\bullet-}$, H_2O_2 , $^\bullet\text{OH}$, and $^1\text{O}_2$ radicals (Tables 1 and 3). The acetone extracts contained higher radical scavenging activity for DPPH, $\text{ABTS}^{\bullet+}$ and ROO^\bullet compared to the juice (Table 1). The ‘B-76’ had the highest abilities to scavenge different reactive oxygen species (Tables 1 and 3). The values for ORAC and $\text{ABTS}^{\bullet+}$ of ‘B-76’ from juice were 68.5 and 2.92 mmol Trolox (TE)/kg fw, respectively. The scavenging capacity for $\text{O}_2^{\bullet-}$, H_2O_2 , $^\bullet\text{OH}$, and $^1\text{O}_2$ in ‘B-76’ fruit juice were 50.4, 2.0, 15.9 and 7.6 mmol ascorbate/kg fw, respectively (Tables 1 and 3). The ED_{50} values ranged from 4.47 to 5.43 mg fresh weight when deerberry were extracted with acetone, while they ranged from 6.48 to 7.40 mg fresh weight without acetone. The fruit of ‘B-76’ also had the greatest scavenging activities against the DPPH radicals with an ED_{50} of 4.47 for acetone extracts (Table 1). A smaller ED_{50} value corresponds to a greater DPPH radical scavenging activity.

Deerberries had high antioxidant enzyme activities as well as the nonenzyme components, ASA and GSH (Table 4). Various activities of antioxidants and oxygen scavenging enzymes were detected in deerberries. Genotype ‘B-76’ deerberry had the highest activities of oxygen scavenging enzymes as well as the nonenzyme components ASA and GSH among all genotypes used in this study. ‘B-76’ deerberry had 22.5 U/mg protein SOD, 27.6 $\Delta\text{A}/\text{mg}$ protein per min G-POD, 164.3 nmol/mg protein per min GSH-POD, 24.2 nmol/mg protein per min AsA-POD, 17.1 nmol/mg protein per min MDAR, 6.8 nmol/mg protein per min DHAR and 5.3 nmol/mg protein per min GR (Table 4). ‘B-76’ also contained 19.4 mmol/kg fw ASA and 40.5 mmol/kg fw GSH (Table 4). Antioxidant capa-

cities were highly correlated to antioxidant enzymes activities. SOD activity was correlated with antioxidant activity with R^2 equal to 0.7553, 0.7425, 0.8288, 0.906 and 0.7397 for ROO^\bullet , $\text{O}_2^{\bullet-}$, H_2O_2 , $^\bullet\text{OH}$, and $^1\text{O}_2$, respectively. The activity of GSH-POD was correlated to antioxidant activity with R^2 equal to 0.9746, 0.9698, 0.9952, 0.9979, and 0.9687 for ROO^\bullet , $\text{O}_2^{\bullet-}$, H_2O_2 , $^\bullet\text{OH}$, and $^1\text{O}_2$, respectively. The R^2 values between G-POD and scavenging capacity for ROO^\bullet , $\text{O}_2^{\bullet-}$, H_2O_2 , $^\bullet\text{OH}$, and $^1\text{O}_2$ were 0.9208, 0.9288, 0.865, 0.7775 and 0.9302, respectively. The correlations coefficients (R^2) between AsA-POD activities and the scavenging capacity for ROO^\bullet , $\text{O}_2^{\bullet-}$, H_2O_2 , $^\bullet\text{OH}$, and $^1\text{O}_2$ were 0.9973, 0.9984, 0.9957, 0.9678 and 0.9981, respectively. The R^2 values for MDAR scavenging capacities on ROO^\bullet , $\text{O}_2^{\bullet-}$, H_2O_2 , $^\bullet\text{OH}$, and $^1\text{O}_2$ were 0.9973, 0.9955, 0.9985, 0.9766 and 0.9951, respectively, and the R^2 for DHAR scavenging capacities on ROO^\bullet , $\text{O}_2^{\bullet-}$, H_2O_2 , $^\bullet\text{OH}$, and $^1\text{O}_2$ were 0.8679, 0.8578, 0.9231, 0.9727 and 0.8556, respectively. The correlation coefficient (R^2)

Table 3

Antioxidant activity against superoxide radicals ($\text{O}_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^\bullet\text{OH}$) and singlet oxygen ($^1\text{O}_2$) in three genotypes of deerberries^{a,b}

	B-59	B-76	SHF3A-3:127	significance ^c
$\text{O}_2^{\bullet-}$	40.6 ± 1.8	50.4 ± 1.2	46.4 ± 1.5	*
H_2O_2	1.8 ± 0.2	2.0 ± 0.3	1.9 ± 0.1	ns
$^\bullet\text{OH}$	13.9 ± 0.8	15.9 ± 0.4	14.9 ± 0.5	*
$^1\text{O}_2$	3.9 ± 0.2	7.6 ± 0.5	6.1 ± 0.4	*

^aData expressed as mean ± standard deviation, $n = 6$.

^bData expressed as mmol ascorbate equivalents per kg fresh weight.

^c*, ns, significant or non-significant, respectively, at $P \leq 0.05$ in the same row.

Table 2

Anthocyanins, phenolic compounds (mg/kg fw) and resveratrol ($\mu\text{g}/\text{kg}$ fw) in three genotypes of deerberries^a

	B-59	B-76	SHF3A-3:127	Significance ^b
Resveratrol	47.9 ± 6	68.5 ± 8	36.2 ± 4	*
Caffeic acid ^c	40.6 ± 2.3	61.2 ± 5.5	48.6 ± 3.6	*
<i>p</i> -coumaric acid ^c	27.7 ± 5.4	33.1 ± 5.2	28.8 ± 4.2	ns
Quercetin 3-galactoside ^d	30.8 ± 2.1	43.4 ± 6.2	26.2 ± 1.8	*
Quercetin 3-arabinoside ^d	2.3 ± 1.5	6.2 ± 1.9	4.5 ± 1.6	ns
Quercetin derivative ^d	7.8 ± 0.7	10.1 ± 4.7	9.2 ± 0.8	ns
Quercetin 3-rhamnoside ^d	77.9 ± 6.4	119.5 ± 10.3	94.7 ± 8.2	*
Kaempferol ^c	32.0 ± 2.7	150.2 ± 6.5	135.2 ± 5.4	*
Cyanidin 3-galactoside ^f	744.2 ± 12.8	987.5 ± 21.4	873.7 ± 17.2	*
Cyanidin 3-glucoside ^f	32.2 ± 3.5	36.3 ± 2.8	38.5 ± 2.2	ns
Cyanidin 3-arabinoside ^f	128.1 ± 10.7	201.0 ± 18.3	161.9 ± 11.2	*
Peonidin 3-glucoside ^f	46.8 ± 2.7	68.6 ± 3.6	90.2 ± 4.5	*

^aData expressed as mean ± standard deviation, $n = 6$.

^b*, ns, significant or non-significant, respectively, at $P \leq 0.05$ in the same row.

^cData expressed as mg caffeic acid equivalents per kg fresh weight.

^dData expressed as mg quercetin equivalents per kg fresh weight.

^eData expressed as mg kaempferol equivalents per kg fresh weight.

^fData of anthocyanidin expressed as mg cyanidin 3-glucoside equivalents per kg fresh weight.

Table 4

Activities of antioxidant enzymes [superoxide dismutase (SOD), guaiacol peroxidase (G-POD), glutathione peroxidase (GSH-POD), ascorbate peroxidase (AsA-POD), monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR)] and non-enzyme antioxidants [ascorbic acid (AsA) and reduced glutathione (GSH)] in three cultivars of deerberries^a

	B-59	B-76	SHF3A-3:127	Significance ^b
SOD (U/mg protein)	13.7±0.4	22.5±1.3	14.1±0.7	*
G-POD (ΔA/mg protein min)	25.7±0.2	27.6±0.1	27.3±0.1	ns
GSH-POD (nmol/mg protein min)	115.9±10.7	164.3±18.2	137.2±13.5	*
AsA-POD (nmol/mg protein min)	14.5±1.9	24.2±2.1	19.9±1.0	*
MDAR (nmol/mg protein min)	12.6±0.8	17.1±1.5	15.0±0.9	*
DHAR (nmol/mg protein min)	6.7±0.5	6.8±0.3	6.5±0.2	ns
GR (nmol/mg protein min)	2.1±0.3	9.6±0.6	5.3±0.7	*
AsA (mmol/kg fw)	14.6.0±1.1	19.4±0.7	18.5±0.6	*
GSH (μmol/kg fw)	26.2±0.4	40.5±2.3	35.4±1.8.	*

^aData expressed as mean±SEM.

^b*, ns, significant or non-significant, respectively, at $P \leq 0.05$ in the same row.

for GR activities and scavenging capacities on oxygen species, ROO^\bullet , $\text{O}_2^{\bullet-}$, H_2O_2 , $^\bullet\text{OH}$, and $^1\text{O}_2$ were 0.9696, 0.9643, 0.9929, 0.991 and 0.9631, respectively. The correlations between scavenging capacities on ROO^\bullet (ORAC values) and AsA content ($R^2 = 0.9362$), $^\bullet\text{OH}$ ($R^2 = 0.8019$), $\text{O}_2^{\bullet-}$ ($R^2 = 0.9432$), H_2O_2 ($R^2 = 0.8848$) and $^1\text{O}_2$ ($R^2 = 0.9446$) were also evident. Genotypes with high antioxidant activities also had high GSH contents. R^2 values between GSH and ROO^\bullet , $\text{O}_2^{\bullet-}$, H_2O_2 , $^\bullet\text{OH}$, and $^1\text{O}_2$ were 0.9362, 0.9432, 0.8848, 0.8019 and 0.9446, respectively.

4. Discussion

Deerberry is only harvested from wild plants and in minor quantities in the US and has thus received little attention for research. However, the results from this study showed that genotypes B-59, B-76 and SHF 3A-3:127 had high sugar and low acid content, high antioxidant content, high free radical scavenging capacity, and high antioxidant enzyme activities.

The SSC for deerberries ranged from 14.7% to 16.8%, which was much higher than strawberries (6.0–9.7%) and blueberries (9.0–10.2%). The TA of deerberries ranged from 0.10% to 0.21% which was lower than strawberries (0.42–0.98) and blueberries (0.38–0.82) (Wang, Zheng & Galletta, 2002; Zheng, Wang, Wang, & Zheng, 2003).

Anthocyanins and phenolics are secondary plant metabolites. They protect the plant against damaging photo-dynamic reactions by quenching the excited state of active oxygen species (Larson, 1988; Lewis, 1993; Rice-Evans & Miller, 1998). In addition to anthocyanins, other phenolic compounds were also present in significant amounts in deerberries. Caffeic acid and *p*-coumaric acid were detected in all three genotypes of deerberries with the highest amounts found in 'B-76'. Flavonols such as quercetin 3-galactoside, quercetin 3-arabinoside, quercetin derivative, quercetin 3-rhamnoside, and kaempferol were also detected in deerberries and 'B-76' again had the highest

amount. Quercetin 3-galactoside, quercetin 3-arabinoside, quercetin derivative, quercetin 3-rhamnoside, and kaempferol serve as effective antioxidants (Pratt, 1992).

Kaempferol and quercetin are potent quenchers of ROO^\bullet , $\text{O}_2^{\bullet-}$ and $^1\text{O}_2$ (Pratt, 1992). Quercetin has higher antioxidant capacity against peroxy radicals (ROO^\bullet) compared to kaempferol (Ratty & Das, 1988). Quercetin and other polyphenols have been shown to play a protective role in carcinogenesis by reducing the bioavailability of carcinogens (Starvic, Matula, Klassen, Downie, & Wood, 1992).

Anthocyanins are potent flavonoid antioxidants that provide health benefits and contribute antioxidant capacity. Free radical scavenging properties of the phenolic hydroxy groups attached to ring structures are responsible for the strong antioxidant properties of the anthocyanins (Rice-Evans & Miller, 1996). Cyanidin 3-galactoside and cyanidin 3-arabinoside were the most important anthocyanins in deerberries. The highest amounts of these anthocyanins were found in 'B-76' among the other genotypes used in this study. Ballington et al. (1988) also reported 'NC 78-8' contained 67.8 % cyaniding 3-galactoside and 17% of cyaniding-3-arabinoside and 'NC 84-31' contained 100% cyaniding 3-galactoside of anthocyanins.

Resveratrol is a polyphenolic compound found in grapes (Adrian, Jeandet, Douillet-Breuil, Tesson, & Bessis, 2000), wine (Siemann & Creasy, 1992), peanuts (Sanders, McMichael, & Hendrix, 2000), cranberries (Wang, Catana, Yang, Roderick, & van Breemen, 2002), and *Vaccinium* berries including blueberries, bilberries and deerberries (Lyons et al., 2003; Rimando et al., 2004). Resveratrol has been shown to have high antioxidant properties (Wang et al., 2002) and to reduce risk of cardiovascular disease (Slater, Odum, & Ashby, 1999). It is also antimutagenic and antiviral (Docherty et al., 1999) and effective in cancer chemoprevention (Lin & Tsai, 1999), and apoptosis induction (Surh et al., 1999). The amounts of resveratrol in three genotypes of deerberry reported in this study were higher than those reported by Rimando et al. (2004). This

may be due to different pre-harvest conditions such as growth temperature, soil condition, moisture, irradiation, soil fertility, and year to year variation. The other possibility for this difference may be due to fruit sample preparation (frozen vs. freeze-dried samples) and analysis procedures. Rimando et al. (2004) had used freeze-dried deerberry fruit samples and gas chromatography-mass spectrometry (GC-MS) for analysis. GC-MS provides excellent sensitivity and specificity, however, the high temperature used at the injector, and ion source might cause partial isomerization or degradation of the samples. The grape had higher resveratrol content (5.7–6.7 mg fw kg⁻¹) compared to the other berry fruits (Adrian et al., 2000; Lyons et al., 2003; Rimando et al., 2004). Deerberries not only possess antioxidant activities against DPPH radicals and ABTS^{•+} radicals, but also have the capacity to scavenge ROO[•], O₂^{•-}, H₂O₂, [•]OH, and ¹O₂. These radicals probably constitute the reactive oxygen species responsible for creating oxidative stress, and may play a role in several chronic diseases (Ames et al., 1993). Cells contain several mechanisms to inactivate these reactive oxygen species and repair or replace damaged cellular molecules in order to maintain cellular homeostasis (Yu, 1994). Different genotypes showed varying degrees of scavenging capacity on different active oxygen species. Among the genotypes tested, 'B-76' had the highest scavenging capacity of active oxygen species.

Antioxidant enzymes have the capacity to lower the free radical burden and neutralize excess free radicals created by stress conditions. GSH-POD, SOD, G-POD, AsA-POD, MDAR, DHAR, GR and the non-enzyme antioxidants, AsA and GSH were all detected in deerberry fruit. SOD is a class of metal-containing proteins, catalysing the dismutation of superoxide radical anions into H₂O₂ and molecular oxygen (Scandalios, 2001). G-POD and AsA-POD are peroxidase enzymes that are found in animal, plant and microorganism tissues, and can catalyse oxidation-reduction between hydrogen peroxide (H₂O₂) and various reductants (Hiraga, Sasaki, Ito, Ohashi, & Matsui, 2001). G-POD is involved in a large number of biochemical and physiological processes. GSH-POD may be responsible for scavenging H₂O₂, catalysing the peroxidation of reduced glutathione (GSH), and forming the oxidized disulfide form of glutathione (GSSG) as a product. AsA-POD is highly specific for ascorbate as the electron donor. Ascorbic acid serves as an excellent antioxidant and plays a fundamental role in the removal of hydrogen peroxide and produces DHAsA. DHAsA is reduced to ascorbic acid by MDAR or DHAR at the expense of NADH and GSH (Halliwell, 1982). GSH has an important function in maintaining cellular redox status (Rennenberg, 1980). GR is a ubiquitous NADPH-dependent enzyme and may be a rate-limiting enzyme for defense against active O₂ toxicity (Gossett, Banks, Millhollon, & Lucas, 1996). Deerberries contained all these antioxidant enzymes, which can regulate free radical activity and can reduce the generation of radicals and prevent cellular and tissue damage in the

human body. Reducing oxidative stress through inducing antioxidative enzyme activity may also suppress the proliferation of tumor cells or inhibit neoplastic transformation (Toyokuni, Okamoto, Yodoi, & Hiai, 1995). In deerberry, 'B-76' had the highest activity of antioxidant enzymes, which were also found to have high antioxidant activity.

Collectively, our data showed that deerberry soluble solids, titratable acids, anthocyanins, and total phenolics vary among cultivars. Deerberry fruit extracts contain potent free radical scavenging capacities for DPPH[•], ABTS^{•+}, ROO[•], [•]OH, H₂O₂, O₂^{•-} and ¹O₂ radicals. Antioxidant capacities in deerberries were highly correlated to their antioxidant enzymes activities. Different cultivars exhibited varying degrees of scavenging capacity and antioxidant enzyme activity. Among the three genotypes, 'B-76' had the highest level of antioxidants and antioxidant enzyme activity. High AsA content and activities of SOD, G-POD, GSH-POD, AsA-POD, MDAR, and DHAR could increase the capacity for free-radical scavenging. The high activity of GR and the high level of GSH could prevent the formation of S–S bonds, therefore providing high antioxidant activity in deerberries. These results indicate that consuming deerberry fruit may be beneficial to human health.

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